Organometric, structural and substructural changes in spleen in terms of experimental colon adenocarcinoma development

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Abstract

Cancer immunology still has many paradoxical questions. One is of the role of the spleen in oncogenesis as well as its own structural reorganization effect on antitumor immunity. Literary data is contradictory and often radically opposite. Therefore, we conducted organometric studies and found out that at the time of pathohistological confirmation of adenocarcinoma in situ in the colon the spleen significantly decreases in size and loses weight. Histologically, deep destructive and degenerative changes in all components of the organ were found: fibrosis, degeneration and destruction of lymphoid tissue, focal zones of destruction in the red pulp. Submicroscopic analysis revealed the presence of lymphocytes with signs of apoptosis in the mantle and periarterial zones. In the white pulp, changes in part of the plasmatic cells were observed: a decrease in the size of the nuclei, damaged GER tubules and mitochondrial cristae. The severity of structural disorders of the spleen increases in the dynamics of the neoplastic lesion development.
Introduction
Cancer is nowadays considered to be the most progressive and disturbing disease posing a threat of mortality despite all advances in medical technology for its diagnosis and curation [1]. Colorectal cancer (CC) is on the top of cancer-killers list among the human population. Since the development of tumors takes around 6–8 months to develop in the DMH/AOM rat model, preneoplastic lesions can be used as biomarkers for evaluating the risk of colon carcinogenesis [2]. The use of preneoplastic lesions as biomarkers was not possible until 1987, when Bird [3] developed a simple, rapid and cheap methodological approach to detecting ACF [3, 4, 5]. In the last decade, additional biomarkers of colon carcinogenesis have been identified. There are tumor markers like carcinoembryonic antigen (CEA), tumor associated antigen L6 and other. However, almost every healthy person can detect a slight increase in these indexes. A significant increase in the concentration of tumor markers is observed in the later stages of the disease, when malignant oncological formation is sufficiently developed. Significant is also the fact that the determination of tumor markers such as enzyme-linked immunosorbent assay (ELISA) and PCR. This procedures are quite expensive, and are implemented in countries with low income only in certain laboratories. According to the published data, a similar situation is no different in the developed countries, because insurance often does not reimburse the cost of expensive diagnostics. This limits the patient and the doctor on an early diagnosis of cancer. Thereby, a universal marker that can detect bowel cancer at an early stage of the process to this day has still not been found and the search for additional cancer indicators is still continued.

To date, considerable attention has been attracted to the inflammation as a key risk factor in colon cancer development [1, 6, 7]. Recent research focused on the changes of the immune system activity at different stages of cancer development. The spleen is the most important immune organ in the body and it contains one-fourth of all of the immune cells in the body. It consists of innate and adaptive immune cells and plays a critical role in the anti-tumor immune response. It contains both tumor-suppressive cells, including natural killer (NK) cells and activated T cells, and tumor-promoting cells, such as regulatory T cells (Tregs), myeloid-derived suppressor cells (MDSCs), and tumor-associated macrophages (TAMs). The spleen cells respond to the pathogens, circulating in blood, and play a crucial role in the immune surveillance [8]. The spleen provides both the innate and adaptive sections of the immune system, allowing an immediate innate reaction to microbial invasion, and an adaptive antigenspecific immune response [9]. The spleen can eliminate nascent tumor cells at the early stages and lead to tumor immune tolerance at later stages of tumor progression. Therefore, it has a paradoxical role in tumor immunology [9-12].

The spleen is usually considered to be an indispensable part of the anti-tumor immune response. Splenic NK cells, as the first line of defense in the initial immune response, may kill mutant cells during tumor onset [13]. The transfection of IL-2 and/or IL-12 genes into the spleen has been reported for the treatment of liver cancer in rats. The mechanism depends on the improvement of NK cell activation and cytokines synthesis [14]. In an SC42 hepatoma murine model, during its early stages, splenic NK cell activity increases, that leads to the suppression of pulmonary metastasis [15]. Splenic T cells are the major effector cells for cytotoxicity against tumor cells [16]. It was established, that splenectomy decreases T cell percentages and promotes W256 carcinosarcoma growth[17]. Also, splenectomy in colon tumor-bearing mice can force liver metastasis by increasing Foxp3 mRNA expression in the liver [18].
However, today there is no unity of scientific judgments about the role of the spleen in carcinogenesis. T. Toge et al. [19] established that the T lymphocytes, which are capable to suppress the anti-tumour activity of the immune system, enter the bloodstream directly from the spleen. K. Noma et al. [20] based on results of conducted research also make a conclusion about the immunosuppressive role of the spleen in cancer of the stomach, which promotes the activation of the oncology process in the stomach. L. Mellemkjaer et al. [21] in a large-scale scientific study, the risk of development of oncopathology in patients with splenectomy due to its traumatic injury (1103 persons) and splenectomy caused by other causes (5212 persons) was assessed. The fact of the development of oncology process was followed by data from the Danish Cancer-Register. The authors of the study concluded that there was no increase in the number of cancer lesions in patients with post-traumatic splenectomy. In their opinion, an increase in the incidence of oncopathology in a group of patients with splenectomy for non-traumatic causes may be due to the progression of the underlying disease or the side effects of its treatment. Literary data interprets the effect of the culture of the spleen cells on the activity of the development of experimental oncopathology in two ways - either the progression of tumor growth, or inhibition. The same situation with the estimation of spatial changes in the spleen - either reducing the size and compaction of the organ tissue, or splenomegaly [22]. The mechanism of the discrete function of the spleen in tumor immunity has not been completely elucidated.

Results
Evaluation of the spleen organometric parameters in dynamics of carcinogenesis
The experimental study showed that the body weight of white rats in the control group increased statistically significantly during 30 weeks of follow-up from (175.9 ± 0.2) g to (231.9 ± 0.3) g (p <0.001).

In the group of animals treated with DMH, the initial body weight was (178.6 ± 0.6) g continued to decrease progressively during all observation periods. At 30 weeks from the start of carcinogen administration, the body weight of the experimental animals was 22.3 % lower than baseline (p<0.01) and 40.1 % (p <0.001) lower than in the control group of animals and was (138.8 ± 0.2) g (Fig.1A).

The mass of the spleen in the group of control animals during all observation periods increased and at 30 weeks was (0.854 ± 0.033) g (compared to (0.605 ± 0.021) g at the beginning of the observation). The spleen mass of white rats with modeled neoplastic process was (0.634 ± 0.031) g at the beginning of the observation. During 30 weeks of the experiment, the spleen mass of animals with DMH lesions decreased statistically significantly. It should be noted that the dynamics of spleen mass decrease in animals with simulated carcinogenesis had certain features: after 30 days of modeling, spleen mass decreased to (0.592 ± 0.031) g and did not change during the next 60 days of observation. Progressive decrease in organ mass was observed starting from 120 days of the experiment. The studied indicator reached its lowest value at 30 weeks of observation and was (0.483 ± 0.019) g, which is 23.8 % lower than the initial level in this group of animals and 43.4 % lower than in animals of the control group (p<0.001) (Fig.1B).
Figure 1. Dynamics of changes in body weight (A) and spleen mass (B) in animals of control and experimental groups.

Determination of mass coefficient (MC), which is used in toxicological studies to assess the condition of internal organs, showed that in animals of the control group, it increased in proportion to the growth of body weight and organ weight. In animals with a simulated neoplastic process, MC decreased, but it should be noted that in the period from 30 to 120 days of observation it was significantly higher than in other observation periods, due to the predominance of weight loss over the loss of spleen mass (Fig. 2).
Figure 2. Mass coefficient in the group of control animals and in the group of animals with induced carcinogenesis in the dynamics of the experiment.

The development of induced neoplastic lesions of the colon in experimental animals was accompanied by a significant change in the spatial characteristics of the spleen. During the entire observation period, the length of the spleen significantly decreased – statistically significant changes were observed after 90 days of DMH administration. At the final point of the experiment (210 days) it was 27.3 % less than the same indicator in control group animals (Fig.3A). The changes in such organometric parameter of the spleen as width differed slightly in the dynamics of the experiment. Thus, a significant decrease in the width of the studied organ was observed only after 150 days of follow-up with further progression and at 30 weeks of the experiment, it was 17.0 % less in comparison with the control animals (Fig.3B).

Analysing the collected data about the changes of such important organometric parameter as spleen thickness, it should be stated, that for the first 90 days it did not differ much from the same indicator in control group rats. However, after 120 days of experiment the thickness of the spleen under the conditions of induced carcinogenesis underwent a significant change in the direction of decrease (by 20.5 %) with its further decrease after 150, 180 and 210 days of observation by 23.1 %, 26.3 % and 42.3 % respectively compared with the same indicator in the control group animals (Fig. 3C).
Figure 3. Changes of the spleen organometric parameters in terms of induced carcinogenesis in the dynamics of the experiment. A. Changes of the spleen length. B. Changes of the spleen width. C. Changes of the spleen sickness.
Microscopical and submicroscopical investigation of the spleen under DMH intoxication

Above-listed data on the changes of organometric parameters of the spleen points on the serious damage of its morphological structure.

Histological examination of the spleen under intoxication by DMH for 30 days showed its typical light-optical structure. Structural and functional connections, namely between the red and white pulp, are clearly defined. Structural rearrangement of the microcirculatory bed of the red pulp comes to the fore. The lumens of the sinusoids are dilated, full-blooded. The wall of the trabecular arteries is swollen; endotheliocytes are desquamated in certain areas.

The boundaries of lymphoid follicles are blurred. Lymphocytes of the mantle zone move to the marginal zone (Fig.4A). The lumen of the central artery is narrowed. Endotheliocytes are exfoliated from the basement membrane, have a tile-like arrangement. The nucleoplasm of endothelial cells is hyperchromic. Small hemorrhages are found on the entire surface of the histological section. The lymph nodes of the white pulp have a different area, but there are enlarged nodules with pronounced zoning. There is low quantity of stroma reticular fibers in the white pulp, they are mainly eccentrically arranged around the central artery, and in the germinal centre they form a small looped net, in which there are lymphocytes and plasma cells.

60 days after the beginning of the experiment, the manifestations of hyperemia of the spleen were less expressed. However, histological examination revealed dilated full-blooded sinusoids of the red pulp. Signs of stasis and edema of the wall of the trabecular arteries and isolated punctate hemorrhages persisted. At the same time, the lumens of the lymphoid nodule vessels did not contain any blood cells. The boundaries of the lymphoid follicles remained blurred. If the lymphocytes in the lymphoid nodules in the previous study were placed compactly, in this period the study traced enlarged intercellular spaces, which indicates their delimitation (Fig.4B).

The nuclei of the lymphocytes of the mantle and marginal zones were intensively stained with haematoxylin and their cross-sectional area was larger in the mantle zone. The lumen of the central artery, in contrast to the manifestations in the first stage of the study, was expanded.

Manifestations of delimphatization after 90 days of intoxication with DMH hydrochloride become even more pronounced. Lymphoid follicles of different size and shape are visible and the boundaries between them and the red pulp are blurred in places, in places clearly contoured.

Edema of the amorphous component of the intercellular substance, thickening of the reticular and collagen fibers of the capsule and trabeculae are established.

The vessels of the organ are significantly changed. The lumens of the central arteries are dilated, the phenomena of endothelial cell desquamation persist. The lumens of the sinusoids are narrowed, along their course there is a significant accumulation of hemosiderophages. Hemosiderin is contained in the form of granules not only in the cytoplasm of macrophages, but also outside them, reproducing the picture of hemosiderosis of the spleen. Fibrin, erythrocyte and mixed thrombi, aggregation of erythrocytes, thrombocytes and leukocytes are found in the wide lumens of many blood vessels due to impaired hemocoagulation in the form of disseminated intravascular coagulation (DIC syndrome). Stasis in sinusoidal hemocapillaries is observed in the microcirculatory tract, which reflects the slowing of blood flow.

By 120 and 150 days of intoxication, the structural manifestations of splenic remodeling correspond to those described above. Histologically, the changes that characterize its dysfunctional structure predominate, namely the manifestations of white pulp delimphatization, red pulp
hemosiderosis with initial signs of sclerosis (Fig.4C). The formation of chronic neoplastic intoxication under the action of DMH leads to the reorganization of the white pulp: it is increased in area, clearly separated from the red pulp due to increased lymphocyte density in the marginal areas of the nodules. On the periphery of the organ and in the white pulp, there was an increase in the density of small, medium lymphocytes, plasma cells and macrophages. In the white pulp, there is an enlightenment of the germinal centers due to a decrease in cell density. In marginal areas, the density of small and medium lymphocytes and plasmatic cells decreases significantly. The clarity of the zonal arrangement of structural components in the nodes is disturbed. Significant dyscirculatory changes in the studied organ were also established. There are dilated and full-blooded vessels with the development of the sludge phenomenon. Microscopically in the red pulp, excessive deposition of blood in the form of accumulation of erythrocytes and hemorrhages is detected. The capsule becomes thin, the trabeculae thicken, and their stromal components are fragmented, located in loose bundles.

Histological research on the 180 and 210 days of intoxication did not differ significantly from the changes described above. During these periods, the portion of the red pulp considerably exceeds the white. It should be noted that in the red pulp the phenomena of collagen formation along the artery and sinusoids increased (Fig.4D), which means that processes of their capillarization took place. In turn, it fixes the process of ischemic alteration of the splenic parenchyma, and stimulates sclerotic changes. The expressed hypostasis and growth of fibers of a connective tissue component of the musculoskeletal system of the spleen is shown by thickening of trabeculae.

Uneven blood supply of red and white pulp is revealed. The vessels in some parts of the spleen are anemic, in others they are overflowing with blood, which reflects congestion. Extensive hemorrhages and blood clots of the vessels of the microcirculatory tract are also present. Intra- and perivascular hemolysis of erythrocytes is noted in part of vascular lumens and red pulp. Focal or local zones of destruction, intravascular and perivascular hemolysis of erythrocytes with accumulation of hemosiderin grains for this term in macrophages are found in red pulp. Red and white pulp is characterized by increased activation of fibroblasts, which leads to thickening of stromal connective tissue and fibrosis. The white pulp shows degeneration and destruction of lymphoid tissue, which is characterized by a decrease in its volume and width of the marginal area. In part of the lymphoid nodules, the germinal centers disappear, along their periphery there is a disorganization of the periarterial lymphoid zones.

The wall of the central artery thickens considerably and, most importantly, the white pulp follicles become small with fuzzy contours of the transition from the mantle zone to the red pulp, and the expansion of intercellular spaces (Fig.4E).
Figure 4. Structural changes of the rats’ spleen under DMH intoxication.

A. The structure of the white and red pulp of the spleen, day 30 of the experiment. Blurred boundaries between the mantle and marginal areas and red pulp. Hematoxylin and eosin staining. Ocular magnification 10X, objective 20X.

B. The structure of the lymphoid nodule of the white pulp of the spleen, day 60 of the experiment. Lymphoid delimitation. Dystrophic changes of endothelial cells and their desquamation. Hematoxylin and eosin staining. Ocular magnification 10X, objective 40X.

C. The structure of the white and red pulp of the spleen, day 120 of the experiment. Loss of clarity of contours between the mantle and marginal areas and red pulp. Delymphatization and different size of lymphoid nodules. Hematoxylin and eosin staining. Ocular magnification 10X, objective 10X.

D. The structure of the red and white pulp of the spleen, day 210 of the experiment. Thickened layers of connective tissue. Clusters of hemosiderophages. Hematoxylin and eosin staining. Ocular magnification 10X, objective 20X.
E. The structure of the red and white pulp of the spleen, day 210 of the experiment. Sclerosis and the predominance of red pulp over white. Lymphoid follicles of different sizes. Delimphatization of lymphoid follicles. Hematoxylin and eosin staining. Ocular magnification 10X, objective 10X.

Submicroscopically, under the conditions of the experiment, lymphocytes with signs of apoptosis were observed in the white pulp of the spleen in the mantle and periarterial zones. Some of these cells in the karyoplasm of the nucleus have large osmophilic, condensed areas, mainly located near the nuclear envelope. There were few organelles in their cytoplasm (Fig.5A).

Lymphocytes with signs of fragmentation of the nucleus into micronuclei are also detected. In the formed micronuclei almost all karyoplasm is filled with condensed chromatin. The cytoplasm looks unstructured, the plasmalemma of the cells is poorly contoured (Fig.5B).

Electron microscopically in the white pulp of the spleen of animals with experimental carcinogenesis, changes in the part of plasma cells are observed. Their nuclei are small, the karyoplasm is filled with heterochromatin, and the perinuclear spaces are expanded. In the cytoplasm there are unevenly thickened tubules of the granular endoplasmic reticulum, osmophilic layered structures, mitochondria with an enlightened matrix and damaged crystas (Fig.5C).

In long-term experimental carcinogenesis, enlargement of macrophages is observed submicroscopically. In their cytoplasm, which has a large area, there are large osmiophilic fragments of phagocytosed cells that were killed and disposed of (Fig.5D).

Figure 5. Substructural changes of the rats’ spleen under DMH intoxication.
A. Submicroscopic changes of the white pulp of the animal's spleen during experimental
carcinogenesis. Nucleus with condensed lumps of heterochromatin (1), cytoplasm of lymphocytes (2). 12 000X

B. Submicroscopic changes of the white pulp of the animal's spleen during experimental carcinogenesis. Nuclear fragmentation into micronuclei (1), homogenized portion of lymphocyte cytoplasm (2). 12 000X

C. Submicroscopic changes of the plasma cell of the white pulp of the animal's spleen during experimental carcinogenesis. Karyoplasm of the nucleus with heterochromatin (1), unevenly thickened tubules of the granular endoplasmic reticulum (2). 12 000X

D. Submicroscopic state of the macrophage of the animal's spleen in experimental carcinogenesis. Nucleus (1), cytoplasm (2), phagocyted material (3). 12 000X

Thus, under the chronic intoxication by DMH remodeling of the spleen of white rats is manifested by the phenomena of delimitation and displacement of the white pulp with the red pulp with the development of sinusoidal capillarization, which stimulates sclerotic changes.

Histological studies of white rats spleen in the dynamics of chronic neoplastic intoxication induced by DMH revealed the development of destructive-degenerative and sclerotic changes of blood vessels, stroma, red and white pulp, the severity of which increased according to the elongation of the observation period.

Discussion

Studies in which experimental tumor is surgically implanted can not illustrate accurately all the changes, that develop in the organism during the whole carcinogenetic cycle. All stages of the tumor formation are accompanied by corresponding changes of cell and humoral immunity, certain nitrooxidative reactions etc.

Thus, we used the prolonged carcinogenesis model, which allowed to establish the nature and severity of spatial changes in the spleen in dynamics of development of induced adenocarcinomatosis of the colon. The decrease in organ mass and its linear characteristics probably occurs due to hypoplasia of the red and white pulp, which requires further histological examination of changes in the structural organization of the studied organ [31].

Given that the spleen is a lymphoid organ that plays an important role in ensuring the immunological reactivity of the organism, it is advisable to study the indicators of cellular and humoral immune systems and their correlation with the immunomorphological status of the organ.

The red pulp is a blood filter that eradicates innate material and damaged erythrocytes. It is also a stowage for iron, erythrocytes and platelets. In rodents, it is a site of hematopoiesis, particularly in fetal and neonatal animals [8]. Thus, it can be assumed, that the destruction of the red pulp can lead to reduced elimination of the pathogens and cellular debris, as well as ageing erythrocytes, from blood, increasing the general intoxication.

The spleen is also the largest secondary lymphoid organ comprising about one-fourth of the body's lymphocytes and instigates immune responses to blood-borne antigens [23-25]. This function is charged to the white pulp that surrounds the central arterioles. The white pulp is constituted of three sub-compartments: the periarteriolar lymphoid sheath (PALS), the follicles, and the marginal zone.

The white pulp consists of the PALS, the follicles, and the marginal zone. As the central arterioles pass into the red pulp, they are surrounded by the PALS composed of lymphocytes and
concentric layers of reticular fibers and flattened reticular cells [26, 27]. The PALS are distributed into the inner PALS and the outer PALS [28-30]. The cells of the inner PALS are mostly CD4+ T-cells, however smaller numbers of CD8+ T-cells can also be present, as well as interdigitating dendritic cells, and migrating B-cells [30]. The outer PALS houses small and medium lymphocytes (both B- and T-cells), macrophages, and, in terms of antigenic stimulation, plasma cells [28, 30]. It is a central site of lymphocyte traffic where the set-up of plasma cells occurs [26, 28]. So, the marginal zone forms a bridge between the innate and adaptive immune response, because the macrophages in this region, which express specific pattern-recognition receptors, can competently go in for blood-borne pathogens. The specific subset of B cells in this area, the marginal-zone B cells, can be activated by these macrophages or can respond directly to blood-borne pathogens, after which they become antigen-presenting cells or IgM-producing plasma cells. Entrance of activated dendritic cells or marginal-zone B cells to the white pulp can pledge an adaptive immune response through activation of T cells, which then drift to the edge of the B-cell follicles and help the B cells [9]. The marginal zone is an exclusive region of the spleen located at the border of the red pulp with the PALS and follicles. Many authors consider it to be a separate section rather than part of the white pulp. However, it is intended to screen the systemic circulation for antigens and pathogens and plays a key role in antigen processing [9, 23]. A group of macrophages, the marginal zone metallophilic macrophages, and the marginal sinus [9, 26, 27], separate the marginal zone from the PALS and follicles. The marginal zone metallophilic macrophages are a distinctive subset of macrophages at the inner margin of the marginal zone neighbouring to the PALS and follicles [9, 26, 28]. The potential functions of the marginal zone metallophilic macrophages are not completely known, but the marginal zone macrophages are significant in elimination of microorganisms and viruses. They express a number of pattern recognition receptors such as toll-like receptors (TLRs) and the macrophage receptor with collagenous structure (MARCO), which are essential in the uptake of various bacteria [9]. The B-cells of marginal zone are an exclusive subdivision of noncirculating B-cells with IgM+/IgD− phenotype as contrasting to follicular B-cells which are IgM+/IgD+ [30].

So we can predict, that established in this study structural changes – disorganization and destruction – in both areas of the spleen may lead to inefficient interaction of cells of the immune system and as a result to loosing an adequate immune reactivity. Probably, these manifestations of remodeling should be regarded as a disruption of adaptation processes in the spleen to the action of oncogenes, which may reflect the manifestation of immunodeficiency [31, 32].

Possessing the received information about probable interrelation between the described functional disturbances and structural changes in the spleen, it is worth considering the usage of cheap and easily accessible methods such as ultrasound examination for predicting the early stage of the neoplastic lesions development.

Materials and methods

Animals

The research was carried out on 84 white outbred male rats with body weight (190 ± 5) g. The animals were retained in standard conditions of vivarium. Body weights and survival were supervised throughout. Experimental animals had free access to drinking water and basal diet ad libitum. All manipulations with animals in this study were conformed in accordance to internationally accepted standards and were approved by the Bioethical Committee of Ternopil National Medical University. All experiments were performed according to the requirements of the
“European Convention for the protection of vertebrate animals used for experimental and other scientific purposes” [34].

**CRC model**

Among all models of chemically induced colon cancer in animals, dimethylhydrazine (DMH) seems to be the most commonly used. The model of DMH induced colon carcinogenesis is well-established and well-appreciated. It has many morphological, biochemical and molecular similarities to human sporadic colorectal cancer [33].

DMH-induced colon adenocarcinoma was modeled by introducing dimethylhydrazine hydrochloride (Sigma-Aldrich Chemie, Japan, series D161802) dissolved in isotonic sodium chloride solution. The chemical carcinogen was administrated subcutaneously into the interscapular region at once a week for 30 weeks. A single dose was 7.2 mg/kg of body weight (based on active substance). Control group animals obtained 0.1 mL of physiological saline with the above frequency and in the same manner [35, 36]. Equal numbers of experimental animals from each group every 30 days, 24 hours after the each last scheduled DMH administration, were deeply anesthetized with Thiopental (50 mg/kg, i.p., Arterium, NUA/3916/01/02) and sacrificed by cervical displacement and exsanguination. At 30 weeks of DMH administration, colon adenocarcinoma in situ was histologically identified in all DMH-treated rats [37].

**Histopathology**

Splenic and colon tissues were harvested from animals and fixed overnight in 10% neutral buffered formalin. The procedure of tissue processing was accomplished in a histoprocessor LOGOSone (Milestone). For histological analysis, all colon, liver and spleen paraffin sections (5 μm thickness) were stained with Hematoxylin and Eosin (H&E) (Biognost) and evaluated with a light microscope Nikon Eclipse Ci. The liver was examined by macroscopy and microscopy to determine the presence or absence of metastasis (absent).

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